

Petrolatum: Barrier repair and antimicrobial responses underlying this “inert” moisturizer

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Background: Petrolatum is a common moisturizer often used in the prevention of skin infections after ambulatory surgeries and as a maintenance therapy of atopic dermatitis (AD). However, the molecular responses induced by petrolatum in the skin have never been assessed.

Objective: We sought to define the cutaneous molecular and structural effects induced by petrolatum.

Methods: Thirty-six healthy subjects and 13 patients with moderate AD (mean SCORAD score, 39) were studied by using RT-PCR, gene arrays, immunohistochemistry, and immunofluorescence performed on control skin, petrolatum-occluded skin, and skin occluded with a Finn chamber only.

Results: Significant upregulations of antimicrobial peptides (S100A8/fold change [FCH], 13.04; S100A9/FCH, 11.28; CCL20/FCH, 8.36; PI3 [elafin]/FCH, 15.40; lipocalin 2/FCH, 6.94, human β -defensin 2 [DEFB4A]/FCH, 4.96; $P < .001$ for all) and innate immune genes (*IL6*, *IL8*, and *IL1B*; $P < .01$) were

observed in petrolatum-occluded skin compared with expression in both control and occluded-only skin. Application of petrolatum also induced expression of key barrier differentiation markers (filaggrin and loricrin), increased stratum corneum thickness, and significantly reduced T-cell infiltrates in the setting of “normal-appearing” or nonlesional AD skin, which is known to harbor barrier and immune defects. **Conclusions:** Petrolatum robustly modulates antimicrobials and epidermal differentiation barrier measures. These data shed light on the beneficial molecular responses of petrolatum in barrier-defective states, such as AD and postoperative wound care. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: Petrolatum, moisturizer, occlusion, patch tests, antimicrobial peptides, innate immunity, atopic dermatitis, barrier, skin surgeries

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Petrolatum, available since 1872,¹ is a widely used moisturizer consisting mainly of long-chain aliphatic hydrocarbons.² It has been shown to decrease transepidermal water loss (TEWL) in healthy³ and irritated^{4,5} human skin. Although petrolatum is formally classified as an occlusive, it has also been described using the broader term “moisturizer,” (a category that includes occlusives, as well as humectants and emollients), which reflects this therapeutic quality.⁶

Petrolatum has been proposed to protect against postambulatory surgical skin infections and is widely used after minor surgical procedures.⁷ A large randomized trial of postoperative ambulatory surgery patients found petrolatum to be equivalent to bacitracin, a topical antibiotic commonly used in the prevention of infections.⁸ Importantly, petrolatum rarely induces allergic contact dermatitis (ACD) reactions⁹ and has never been reported to cause contact anaphylaxis, whereas bacitracin was shown to induce ACD in up to 13% of patients¹⁰ and has caused contact anaphylaxis in several cases.¹¹⁻¹⁵ However, the molecular changes induced by petrolatum are unknown.

Lesional skin of patients with atopic dermatitis (AD) exhibits immune and barrier defects with increased penetration of small molecules.¹⁶⁻¹⁹ This results in increased prevalence of ACD and microbial colonization/infection in the population with AD.^{20,21} Despite similar bacterial colonization in patients with AD and those with psoriasis,²² another common inflammatory skin disease, significantly higher rates of skin infection were observed only in patients with AD.²³ These differences were postulated to result in part from significantly lower antimicrobial peptide (AMP) responses in AD lesions compared with psoriatic lesions.^{22,24} In adult and pediatric patients with AD, moisturizers have been shown to improve AD disease severity,²⁵ TEWL,²⁶ and

Abbreviations used

ACD:	Allergic contact dermatitis
AD:	Atopic dermatitis
AHR:	Aryl hydrocarbon receptor
AMP:	Antimicrobial peptide
DC:	Dendritic cell
DEG:	Differentially expressed gene
FCH:	Fold change
FLG:	Filaggrin
HBD2:	Human β -defensin 2
H&E:	Hematoxylin and eosin
IHC:	Immunohistochemistry
LCN2:	Lipocalin 2
LOR:	Loricrin
PI3:	Peptidase inhibitor 3/elafin
QC:	Quality control
SC:	Stratum corneum
TEWL:	Transepidermal water loss

skin capacitance,²⁷ as well as to reduce rates of *Staphylococcus aureus* colonization.²⁸ Emollients have recently been shown to effectively prevent AD development in high-risk newborns.^{29,30}

This study aims to uncover the molecular responses triggered by petrolatum application that can improve clinical and barrier measures, ultimately reducing infections in patients with barrier-disrupted conditions, such as AD, and after cutaneous surgeries. In 2 separate cohorts totaling 49 patients, we detected significant upregulation of key AMPs and innate immune genes in biopsy specimens from skin occluded with petrolatum compared with occlusion alone and control (or nonlesional AD) skin. Occlusion with petrolatum also resulted in altered epidermal structure and increased expression of terminal differentiation proteins, including filaggrin (FLG) and loricrin (LOR), which were particularly evident when petrolatum was applied to “normal-appearing” or nonlesional AD skin.

METHODS

Patients' characteristics and skin samples

This study included 2 cohorts under institutional review board–approved protocols. The first cohort included 29 patients (18 female/11 male patients; age, 19–62 years [median, 38 years]; 13 with AD/16 without AD; mean SCORAD score, 39). These patients had petrolatum (“White petrolatum”; Dynarex, Israel; NDC #67777-211-01) applied for 48 hours under occlusion with a Finn chamber, with biopsy specimens taken at 72 hours (24 hours after removal of petrolatum). A total of 2 biopsy specimens were obtained from this cohort: petrolatum-occluded and control (nonoccluded or nonlesional in patients with AD) skin. In patients with AD, all patches were applied on uninvolved skin; lesional/eczematous skin biopsies were not involved in this study. The petrolatum used in this study contained white petrolatum USP 100% wt/wt. White petrolatum often contains small amounts of antioxidants to prevent discoloration but does not contain antimicrobial preservatives.

To evaluate occlusion effects, as well as to determine the time point of maximal gene induction, we enrolled a second cohort including 20 healthy volunteers (8 female/12 male subjects; age, 18–80 years [median, 50.5 years]). These subjects had biopsy specimens taken from control skin, skin occluded with petrolatum under a Finn chamber, and skin undergoing occlusion alone (under a Finn chamber). For assessing the time point of maximal gene induction, the first 5 volunteers in the second cohort had 5 biopsies performed: control, occlusion-only and occlusion with petrolatum at 48 hours, and occlusion-only and occlusion with petrolatum at 72 hours skin. After analysis of preliminary kinetic data and to minimize the number of skin biopsies, the

remaining 15 volunteers had only 3 biopsies: control skin, occlusion at 72 hours, and petrolatum at 72 hours (a flow chart of the 2 cohorts is shown in Fig 1).

Immunostaining and immunofluorescence

Immunohistochemistry (IHC) was performed on frozen tissue sections by using anti-human mAbs against S100A8/A9, lipocalin 2 (LCN2), CCL20, FLG, and LOR (see Table E1 in this article's Online Repository at www.jacionline.org). Immunofluorescence staining for neutral lipids with Nile Red was performed, as previously reported.^{31,32} Epidermal thickness and positive cells per millimeter were quantified for IHC with ImageJ V1.42 software (National Institutes of Health, Bethesda, Md), and immunofluorescence was imaged with MetaView software (Visitron Systems, Puchheim, Germany).³³ For further details, see the Methods section in this article's Online Repository at www.jacionline.org.

Quantitative RT-PCR and gene arrays

RNA was extracted for RT-PCR and gene arrays with EZ-PCR Core Reagents (Life Technologies, Grand Island, NY), and custom primers were generated (see Table E2 in this article's Online Repository at www.jacionline.org for primers and probes).^{16,24,34–38} Expression values were normalized to human acidic ribosomal protein (*hARP*). Human HGU133Plus2.0 GeneChip probe arrays (Affymetrix, Santa Clara, Calif) were used for gene arrays.^{16,24,34–38} Total RNA was extracted with the Qiagen miRNeasy Mini Kit (Qiagen, Valencia, Calif), and DNA was removed with the Qiagen RNase-free DNase Set. Total RNA (50 ng) was reverse transcribed and amplified with Ovation Whole Blood Solution from NuGen (San Carlos, Calif). The labeled target was fragmented and hybridized to probe arrays by using the Encore Biotin Module from NuGen. For further details, see the Methods section in this article's Online Repository.

Statistical analysis

The 2 previously described cohorts (cohort 1, 29 subjects; cohort 2, 20 subjects) were analyzed separately. Quality control (QC) of microarray gene expression was carried out with the standard QC metrics from Quality Control R package. Expression values from arrays and RT-PCR were modeled by using linear mixed-effect models from the *limma* and *nlme* packages in R, respectively. For microarray data, differentially expressed genes (DEGs) in petrolatum were identified by the significance of moderated *t* statistics after Benjamini-Hochberg adjustment for multiple hypotheses and with a fold change (FCH) of greater than 2. Analysis of RT-PCR gene expression consisted of fitting the mixed-effects model followed by pairwise comparisons among skin types: petrolatum-occluded, occluded-only, and normal skin (cohort 2). The significance of the differences was displayed in bar plots. The subgroup analysis for patients with and without AD (cohort 1) was carried out by including in the mixed-effects model skin type (petrolatum-occluded or normal skin), AD personal history (yes/no), and their interaction as fixed effects. A random intercept was adjusted for each subject. The same approach used for RT-PCR was applied to IHC counts (cohort 1) under the assumption of normality. Estimating contrasts by using restricted maximum likelihood assessed the significance of the differences between specified groups. Heat maps were built based on the McQuitty algorithm and Euclidean distances. More specific details can be found in the Methods section this article's Online Repository.

RESULTS

Our study included 2 cohorts. The first contained 29 subjects who had biopsy specimens taken after 72 hours from control skin (or nonlesional skin for those with AD) and skin occluded with petrolatum for 48 hours. The second cohort included 20 healthy volunteers with biopsy specimens taken

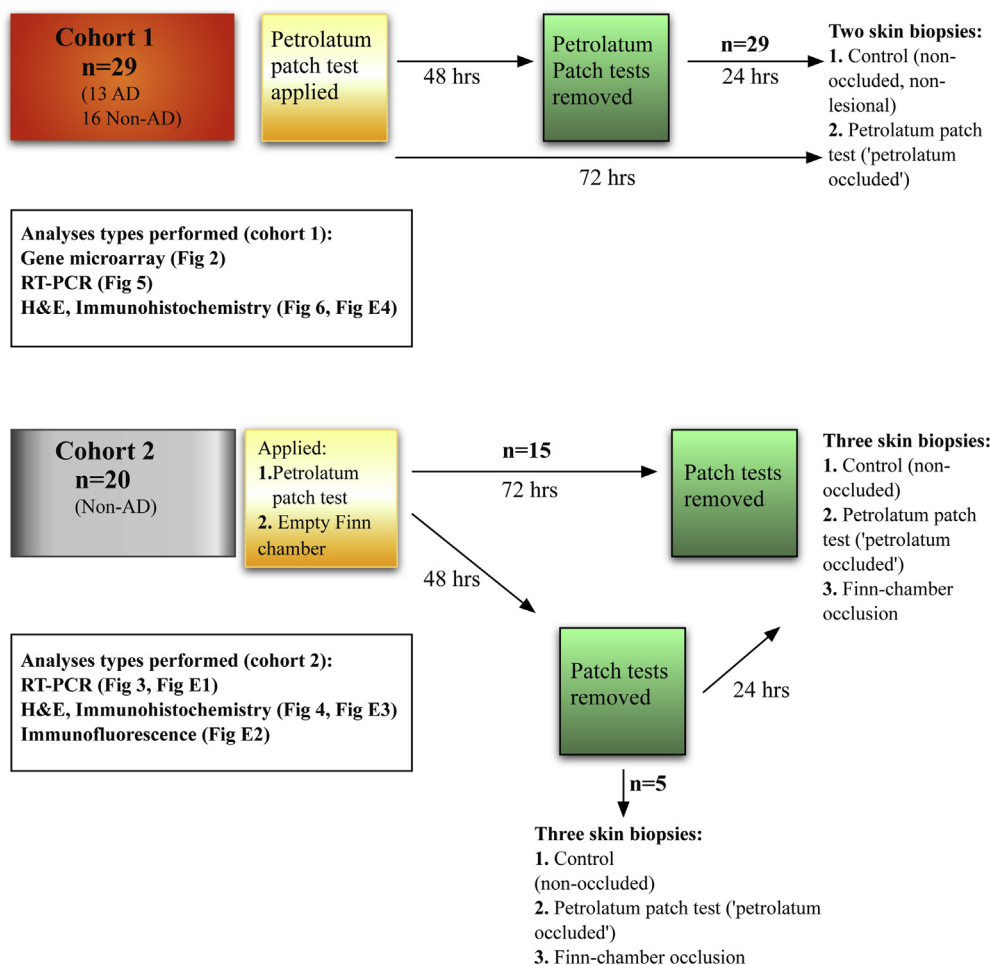


FIG 1. Study design. Two cohorts were enrolled in this study. Cohort 1 included 29 (13 patients with AD and 16 subjects without AD) subjects, and cohort 2 included 20 healthy volunteers. Patch tests were applied for 48 hours in cohort 1 and 72 hours in cohort 2, and biopsy specimens were obtained at 72 hours. Five subjects from cohort 2 had another 3 biopsy specimens taken at 48 hours.

after 72 hours from control, occlusion with petrolatum, and occlusion-only sites (Fig 1).

Occlusion with petrolatum induces tissue expression of innate immune genes

To investigate the general effects of petrolatum (in both AD and non-AD skin), we first compared control skin versus petrolatum patch-tested skin in cohort 1. Gene-expression profiling detected 51 DEGs in petrolatum-occluded compared with control skin (FCH, >2; false discovery rate, <0.05). The DEGs in petrolatum-occluded compared with control skin are presented in a heat map (Fig 2 and Table I).

Several IL-17- and IL-22-regulated AMPs²⁴ were significantly induced by petrolatum. These include human β -defensin 2 (HBD2)/DEFB4A (FCH, 4.96), LCN2 (FCH, 5.42), peptidase inhibitor 3/elafin (PI3; FCH, 4.94), S100A8 (FCH, 2.02), S100A9 (FCH, 7.19), CXCL1 (FCH, 3.25), and CXCL2 (FCH, 2.61; $P < .05$ for all). Certain T-cell and dendritic cell (DC) markers showed mostly minor increases in petrolatum-occluded skin (CD2, CD28, CD1b, and CCL19, a ligand of CCR7 that marks lymphoid structures in skin).³⁹

Petrolatum upregulates AMPs more than occlusion alone

To investigate whether genomic effects are specifically related to petrolatum rather than occlusion, we recruited an additional cohort of 20 healthy volunteers (cohort 2, Fig 1) from whom petrolatum-occluded, occlusion-only, and control skin biopsy specimens were obtained.

Fig 3 presents modulation of the IL-17/IL-22-induced AMPs and selected immune genes by using RT-PCR in skin biopsy specimens from petrolatum under occlusion, occlusion-only, and control skin. Although some significant increases were observed with occlusion alone, occlusion with petrolatum induced more extensive and significant increases in AMP (ie, S100A7/A8/A9/A12, LCN2, elafin/PI3, CCL20, and cathelicidin/LL37; $P < .05$ for all; Fig 3) and innate immune gene (ie, *IL1B*, *IL6*, *IL8*, and *TNFA*; $P < .01$ for all) levels (Fig 3). Significant upregulation of *IL17*, *IL22*, and both the p19 and p40 subunits of *IL23*, a known activator of the T_H17 pathway,^{40,41} was observed in petrolatum compared with both occluded and control skin, supporting our microarray data from the initial cohort. This is in contrast to *IFNG* (Fig 3), which showed similar expression ($P > .1$) in control, occlusion-only, and petrolatum-occluded skin.

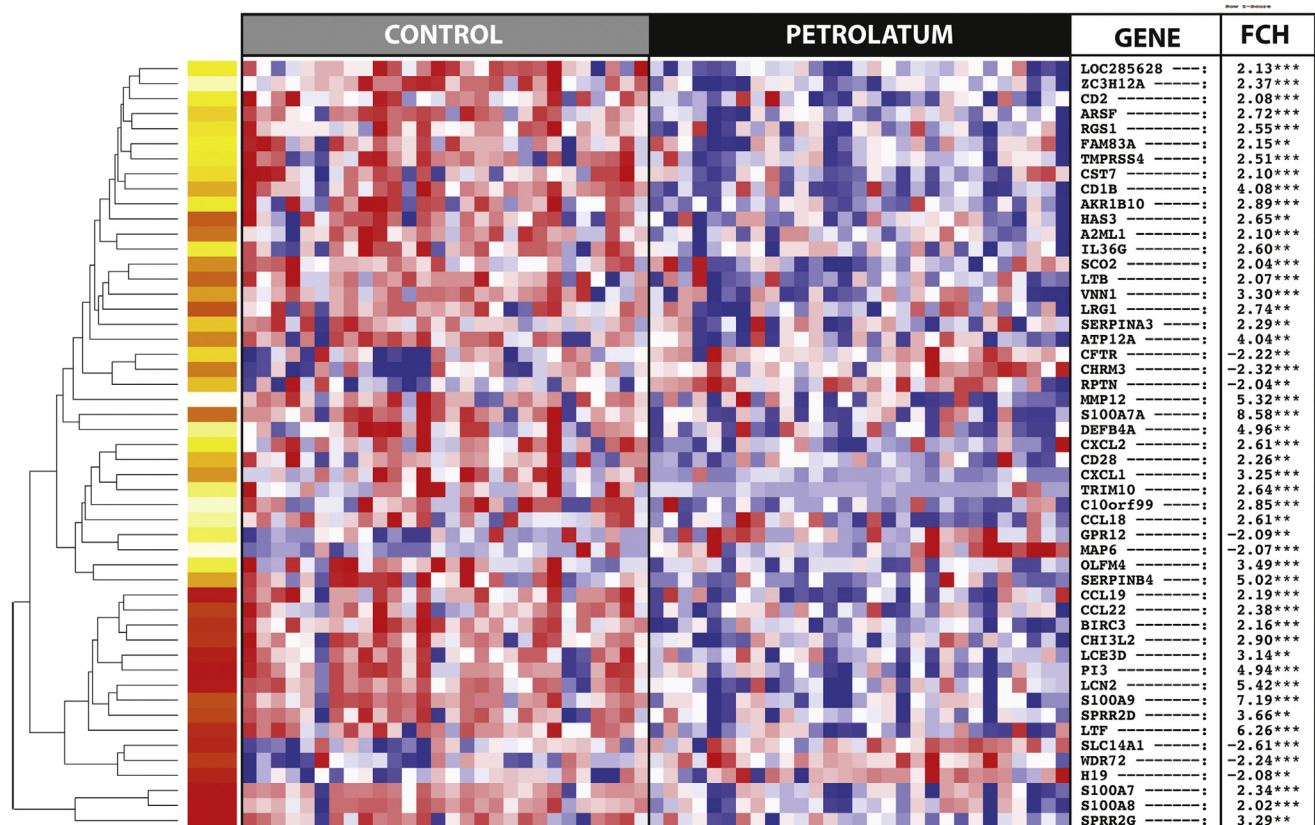


FIG 2. Unsupervised hierarchic clustering of DEGs across petrolatum-occluded and control skin samples (red, upregulated; blue, downregulated). Expression of the AMPs HBD2/DEFB4A, LCN2, elafin/PI3, S100A8, S100A9, CXCL1, and CXCL2 was increased significantly by petrolatum occlusion. The right column shows FCHs of petrolatum compared with control values. ** $P < .05$ and *** $P < .01$.

In the first cohort petrolatum occlusion occurred for 48 hours, with biopsies at 72 hours, whereas in the second cohort we performed a preliminary time-effect study on the first 5 recruited subjects to evaluate for the highest gene inductions. Although some effects were present at 48 hours, maximal induction of AMPs was observed at 72 hours of occlusion with petrolatum (see Fig E1 in this article's Online Repository at www.jacionline.org). Therefore in the additional 15 patients in this cohort, all subsequent biopsy specimens were taken at the 72-hour time point only.

Increased AMP protein expression determined by using IHC

Hematoxylin and eosin (H&E) staining and IHC for select AMPs (S100A8/A9, LCN2, and CCL20) was performed in control, occluded-only, and petrolatum-occluded skin biopsy specimens obtained from patients in cohort 2.

Both occluded and petrolatum-occluded compared with control skin demonstrated thickening of the stratum corneum (SC), as seen by using H&E in Fig 4, A-C, and Fig E2, A, in this article's Online Repository at www.jacionline.org, with a nearly 2-fold increase in thickness seen with petrolatum compared with control skin (41 μ m in petrolatum vs 32 μ m in occlusion vs 24 μ m in control, $P < .01$ for all comparisons; see Fig E2, A). Nile Red immunofluorescence staining for neutral lipids showed widening of the spaces between lamellar bodies most

strikingly with petrolatum occlusion, probably accounting for the thickened SC observed with H&E staining (see Fig E2, B-D).

Increased epidermal staining with the antimicrobial markers S100A8/A9, LCN2, and CCL20 was observed only in petrolatum-occluded skin (Fig 4, D-L) and was most noticeable for S100A8/A9 (Fig 4, D-F).

Neutrophil counts were measured by using neutrophil elastase staining, with no significant increases in neutrophil counts in either occluded-only or petrolatum-occluded skin compared with control skin to rule out infection (rather than colonization) as a potential inducer of AMPs in petrolatum-occluded skin (see Fig E3 in this article's Online Repository at www.jacionline.org).

Differential genomic inductions by petrolatum in patients with AD versus subjects without AD

Because cohort 1 included both patients with AD and subjects without AD ($n = 29$, Fig 1), we sought to evaluate whether petrolatum exerts differential effects in these groups. Thus we compared gene and protein expression separately among control (nonoccluded, nonlesional) and petrolatum-occluded skin in both populations without and with AD. Control skin in the AD group refers to nonlesional skin. Control skin was very different in the 2 populations. In patients with AD, there were large increases in expression of many T_H2 pathway genes (ie, *IL4*, *IL13*, *IL10*, and *CCL26*). AMP (S100A8-9, LCN2, and PI3) expression

TABLE I. DEGs in petrolatum versus control skin through gene arrays

Probe set	Symbol	Gene name	IgFCH_ Petrolatum	FCH_ Petrolatum	pvals_ Petrolatum	fdrs	StatusFCH2FDR0.05_ Petrolatum
1553505_at	<i>A2ML1</i>	Alpha-2-macroglobulin-like 1	1.07	2.1	1.28E-05	0.00455785	1
206561_s_at	<i>AKR1B10</i>	Aldo-keto reductase family 1, member B10 (aldose reductase)	1.53	2.89	4.89E-07	0.00057312	1
214490_at	<i>ARSF</i>	Arylsulfatase F	1.44	2.72	1.01E-08	6.10E-05	1
207367_at	<i>ATP12A</i>	ATPase, H+/K+ transporting, nongastric, alpha polypeptide	2.01	4.04	5.45E-05	0.01081601	1
210538_s_at	<i>BIRC3</i>	Baculoviral IAP repeat containing 3	1.11	2.16	9.51E-13	3.46E-08	1
227736_at	<i>C10orf99</i>	Chromosome 10 open reading frame 99	1.51	2.85	3.42E-05	0.00816811	1
209924_at	<i>CCL18</i>	Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	1.39	2.61	0.00013771	0.01750246	1
210072_at	<i>CCL19</i>	Chemokine (C-C motif) ligand 19	1.13	2.19	2.27E-08	9.17E-05	1
207861_at	<i>CCL22</i>	Chemokine (C-C motif) ligand 22	1.25	2.38	5.35E-06	0.00252604	1
206749_at	<i>CD1B</i>	CD1b molecule	2.03	4.08	2.62E-11	4.76E-07	1
205831_at	<i>CD2</i>	CD2 molecule	1.05	2.08	1.22E-06	0.00106969	1
206545_at	<i>CD28</i>	CD28 molecule	1.18	2.26	9.38E-05	0.01483149	1
205043_at	<i>CFTR</i>	Cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7)	-1.15	-2.22	9.15E-05	0.01466372	-1
213060_s_at	<i>CHI3L2</i>	Chitinase 3-like 2	1.54	2.9	9.61E-08	0.00018377	1
214596_at	<i>CHRM3</i>	Cholinergic receptor, muscarinic 3	-1.21	-2.32	2.63E-05	0.00703566	-1
210140_at	<i>CST7</i>	Cystatin F (leukocystatin)	1.07	2.1	1.48E-05	0.00489151	1
204470_at	<i>CXCL1</i>	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	1.7	3.25	8.69E-08	0.0001832	1
209774_x_at	<i>CXCL2</i>	Chemokine (C-X-C motif) ligand 2	1.38	2.61	4.73E-06	0.00229236	1
207356_at	<i>DEFB4A</i>	Defensin, beta 4A	2.31	4.96	0.00010607	0.01570483	1
239586_at	<i>FAM83A</i>	Family with sequence similarity 83, member A	1.11	2.15	0.00019116	0.02153566	1
1559603_at	<i>GPR12</i>	G protein-coupled receptor 12	-1.07	-2.09	5.45E-05	0.01081601	-1
224997_x_at	<i>H19</i>	H19, imprinted maternally expressed transcript (non-protein coding)	-1.06	-2.08	0.00027757	0.02634261	-1
223541_at	<i>HAS3</i>	Hyaluronan synthase 3	1.4	2.65	8.04E-05	0.01365243	1
220322_at	<i>IL36G</i>	Interleukin 36, gamma	1.38	2.6	0.00011757	0.0164361	1
224328_s_at	<i>LCE3D</i>	Late cornified envelope 3D	1.65	3.14	5.40E-05	0.01081601	1
212531_at	<i>LCN2</i>	Lipocalin 2	2.44	5.42	2.11E-07	0.00033392	1
232504_at	<i>LOC285628</i>	Uncharacterized LOC285628	1.09	2.13	5.32E-08	0.0001522	1
228648_at	<i>LRG1</i>	Leucine-rich alpha-2-glycoprotein 1	1.45	2.74	5.92E-05	0.01137823	1
207339_s_at	<i>LTB</i>	Lymphotoxin beta (TNF superfamily, member 3)	1.05	2.07	5.61E-07	0.00062026	1
202018_s_at	<i>LTF</i>	Lactotransferrin	2.65	6.26	1.35E-08	7.03E-05	1
235672_at	<i>MAP6</i>	Microtubule-associated protein 6	-1.05	-2.07	9.18E-06	0.0036279	-1
204580_at	<i>MMP12</i>	Matrix metalloproteinase 12 (macrophage elastase)	2.41	5.32	5.63E-07	0.00062026	1
212768_s_at	<i>OLFM4</i>	Olfactomedin 4	1.8	3.49	1.31E-05	0.0046275	1
41469_at	<i>PI3</i>	Peptidase inhibitor 3, skin-derived	2.3	4.94	4.90E-08	0.0001522	1
202988_s_at	<i>RGS1</i>	Regulator of G-protein signaling 1	1.35	2.55	2.64E-07	0.00038338	1
1553454_at	<i>RPTN</i>	Repetin	-1.03	-2.04	0.00014025	0.01764038	-1
205916_at	<i>S100A7</i>	S100 calcium binding protein A7	1.22	2.34	2.04E-07	0.00033392	1
232170_at	<i>S100A7A</i>	S100 calcium binding protein A7A	3.1	8.58	5.88E-07	0.00062913	1
214370_at	<i>S100A8</i>	S100 calcium binding protein A8	1.02	2.02	8.85E-08	0.0001832	1
203535_at	<i>S100A9</i>	S100 calcium binding protein A9	2.85	7.19	4.33E-09	3.15E-05	1
205241_at	<i>SCO2</i>	SCO2 cytochrome c oxidase assembly protein	1.03	2.04	2.92E-05	0.00742997	1
202376_at	<i>SERPINA3</i>	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	1.2	2.29	0.00053038	0.03658212	1
211906_s_at	<i>SERPINB4</i>	Serpin peptidase inhibitor, clade B (ovalbumin), member 4	2.33	5.02	6.75E-06	0.00285278	1
229151_at	<i>SLC14A1</i>	Solute carrier family 14 (urea transporter), member 1 (Kidd blood group)	-1.38	-2.61	7.63E-06	0.00315037	-1
208539_x_at	<i>SPRR2D</i>	Small proline-rich protein 2D	1.87	3.66	0.00013187	0.01736658	1
236119_s_at	<i>SPRR2G</i>	Small proline-rich protein 2G	1.72	3.29	5.26E-05	0.01075143	1
218960_at	<i>TMPRSS4</i>	Transmembrane protease, serine 4	1.33	2.51	1.46E-05	0.00489151	1
221627_at	<i>TRIM10</i>	Tripartite motif containing 10	1.4	2.64	1.43E-05	0.00487377	1
205844_at	<i>VNN1</i>	Vanin 1	1.72	3.3	2.72E-06	0.00161902	1
227174_at	<i>WDR72</i>	WD repeat domain 72	-1.16	-2.24	2.45E-07	0.00037126	-1
218810_at	<i>ZC3H12A</i>	Zinc finger CCH-type containing 12A	1.25	2.37	8.98E-11	1.09E-06	1

FDR, False discovery rate.

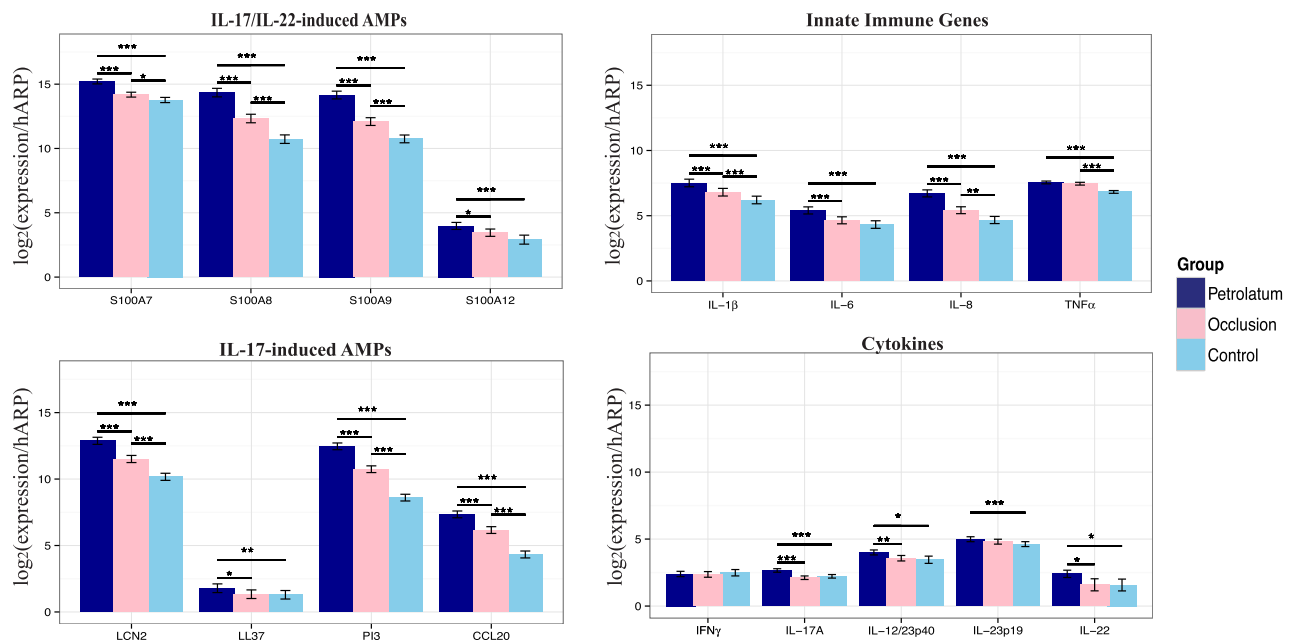


FIG 3. Expression of AMPs, innate immune genes, and major cytokines, as determined by using RT-PCR, in control, occlusion-only, and occlusion with petrolatum skin. Mean expression estimates normalized to *hARP* are represented in a ($\log_2[\text{expression}/hARP]$) scale. Data were grouped according to the major immune pathways represented. Increased expression of significant AMPs, innate immune genes, and cytokines was observed with petrolatum occlusion. *P* values are indicated if significant. **P* < .1, ***P* < .05, and ****P* < .01.

levels were largely similar in non-AD and nonlesional AD skin (Fig 5, A). Furthermore, we evaluated modulation of genomic expression in the non-AD and AD groups compared with their individual control subjects. Overall, similar trends of genomic induction were observed in both groups, but greater increases in AMP (S100A7-9, LCN2, CCL20, and PI3) and immune gene (*IL12/IL23p40*, *IL23p19*, *IL22*, *IFNG*, and *IL17F*) levels were seen in subjects without AD (Fig 5, B). Importantly, no further increases of T_H2 markers were observed with petrolatum occlusion in patients with AD (Fig 5, C).

Occlusion with petrolatum improves epidermal differentiation

To assess epidermal differentiation changes, we performed staining with H&E and the terminal differentiation markers FLG and LOR on petrolatum-occluded and unoccluded skin of patients with AD and subjects without AD (Fig 6).

As evident by using H&E staining (Fig 6, A-D), nonlesional AD compared with non-AD skin displayed parakeratosis and focal disruption of the granular layer. Petrolatum-occluded AD skin demonstrated a renewed continuity of the granular layer and restoration of orthokeratosis. Similarly, FLG and LOR expression was inconsistent and pale in nonlesional AD skin, with intensified and continuous expression after petrolatum occlusion (Fig 6, G, H, K, and L). Non-AD skin also showed increased expression of FLG and LOR in the petrolatum-occluded compared with control skin (Fig 6, E, F, I, and J), but the change was less evident than in the AD group (Fig 6, E-L).

Suppression of cellular infiltrates after petrolatum occlusion in patients with AD

We measured infiltrates of $CD3^+CD8^+$ T cells and $CD1c^+$ DCs in nonlesional and petrolatum-occluded skin of patients with AD and subjects without AD (see Fig E4 in this article's Online Repository at www.jacionline.org). Significant reductions in $CD3^+$ and $CD8^+$ T-cell counts and $CD1c^+$ DC counts with petrolatum were observed mostly (except $CD8^+$) in AD but not in non-AD skin when compared with respective control values (*P* < .05 for all).

DISCUSSION

This study is the first to identify alterations in AMP expression with the application of petrolatum, a substance previously presumed to have no activity in the nucleated layers of the epidermis.⁵ Our findings provide novel insights into the molecular responses induced by an over-the-counter moisturizer frequently used by the public for everything from routine wound care to epistaxis.⁴²

It has been estimated that 14 million skin excisions or biopsies are performed annually in the United States.⁴³ After a pivotal randomized controlled trial showing equivalent postoperative infection rates for petrolatum and topical antibiotic ointment in ambulatory surgery patients,⁸ 69.4% of Mohs surgeons recommend petrolatum for wound care after routine procedures.⁷ This recommended change in practice is due to several factors, such as higher cost,²² increased rates of ACD,⁴⁴ and even cases of contact anaphylaxis¹¹⁻¹⁵ with use of topical antibiotic ointments, such as bacitracin. The induction of various AMPs, innate immune genes, and restoration of barrier responses by

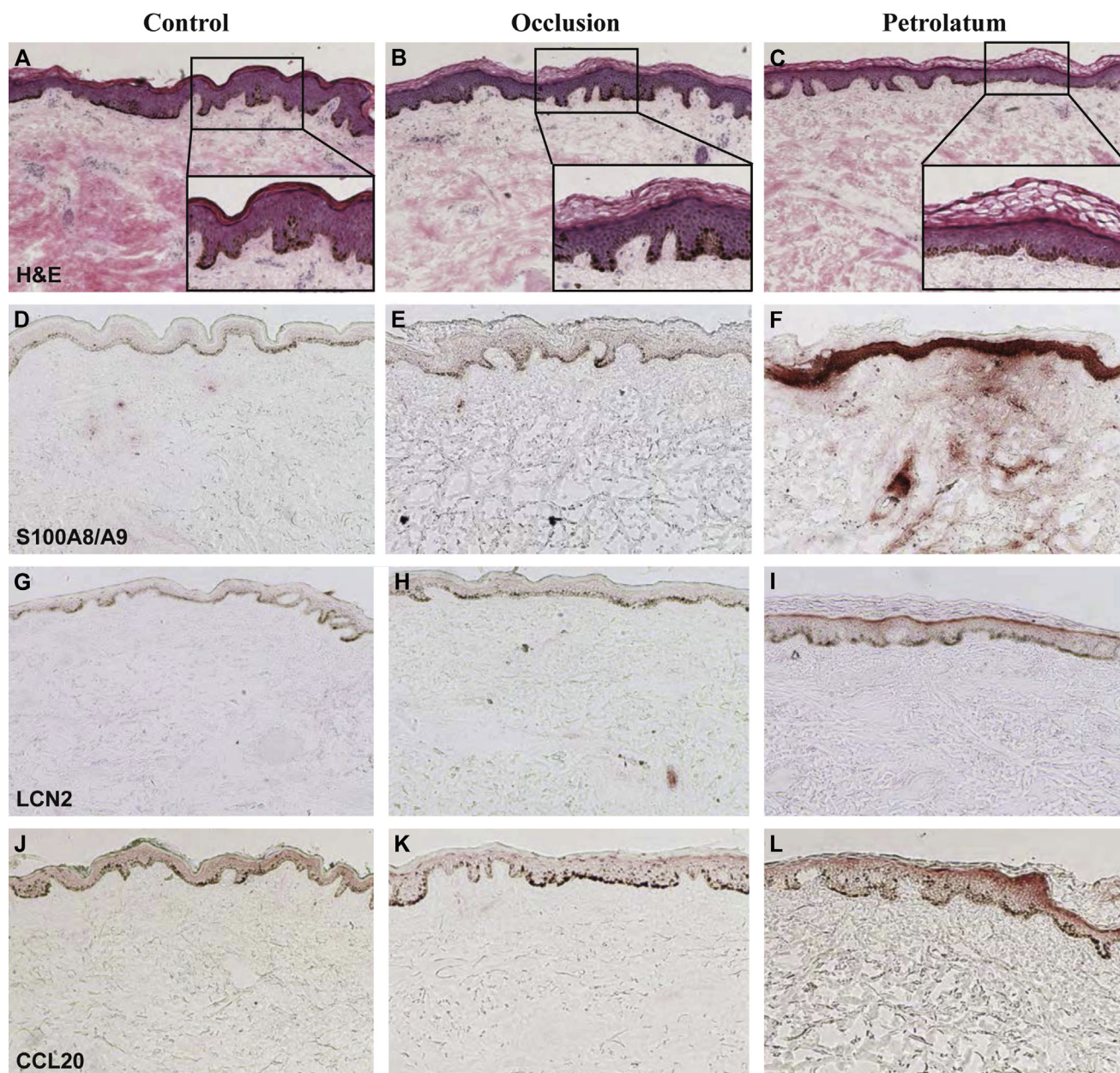


FIG 4. Representative H&E (A-C) and IHC staining of S100A8/A9 (D-F), lipocalin/LCN2 (G-I), and CCL20 (J-L) in control, occluded, and petrolatum-occluded skin samples, demonstrating increased expression of AMPs in petrolatum-occluded skin (histologic magnification $\times 10$). Note also increased thickness of the SC in H&E-stained samples (Fig 4, A-C), with intercellular clefts most notable in petrolatum-occluded skin (Fig 4, C).

petrolatum, as shown in this study, provides a plausible explanation for its efficacy in reducing postoperative infections.

In general, most AMPs are cationic, with an amphipathic structure that allows for interaction with microbial membranes, leading to pore formation and release of cytosolic components.⁴⁵⁻⁴⁷ This mechanism is used by most petrolatum-induced AMPs in our study, including LL-37⁴⁸ and HBD2.^{49,50} LL-37 is one of the most well-characterized AMPs,⁵¹ forming an α -helix to disrupt both bacterial membranes and viral envelopes.^{48,52} Its precursor, cathelicidin, was recently shown to be upregulated in dermal adipocytes on infection with *S aureus*,⁵³ pointing to its importance in innate defense against this

pathogen.^{54,55} Consistent with our results, LL-37 also upregulates innate immune genes, including *IL6*, *IL8*, and *IL10*.⁴⁸ Conversely, HBD2/DEFB4A has potent activity against gram-negative bacteria, including *Escherichia coli* and *Pseudomonas aeruginosa*, but is less effective against gram-positive organisms.⁵⁶ Additionally, calprotectin, the antimicrobial heterodimeric complex formed by S100A8 and S100A9, exerts effects against *S aureus*, *Staphylococcus epidermidis*,⁵⁷ gram-negative bacteria (*E coli*, *Klebsiella* species, and *Capnocytophaga sputigena*),^{57,58} *Borrelia burgdorferi*,⁵⁹ and *Candida albicans*.⁶⁰ Our results show that levels of the S100A8/A9 complex are remarkably increased in petrolatum-occluded skin,

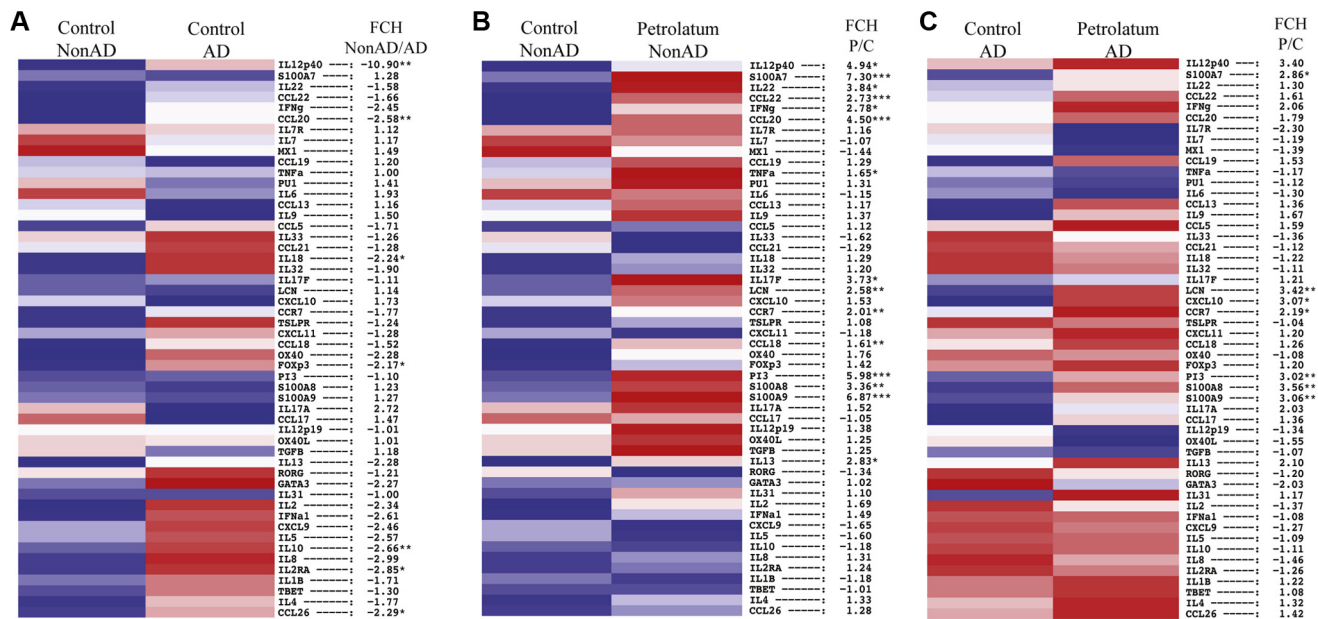


FIG 5. Heat map showing average RT-PCR expression values for control and petrolatum AD and non-AD groups. **A**, Mean RT-PCR expression values in control non-AD and control AD skin. Large T_H2 pathway gene (ie, *IL4*, *IL13*, *IL10*, and *CCL26*) expression increases in control skin were observed in patients with AD. **B** and **C**, Mean RT-PCR expression values in control and petrolatum non-AD (Fig 5, **B**) and AD (Fig 5, **C**) skin, showing greater increases in AMP (*S100A7-9*, *LCN2*, *CCL20*, and *PI3*) and immune gene (*IL12/IL23p40*, *IL23p19*, *IL22*, *IFNG*, and *IL17F*) expression in subjects without AD. FCH values are shown in the columns to the right. * $P < .1$, ** $P < .05$, and *** $P < .01$.

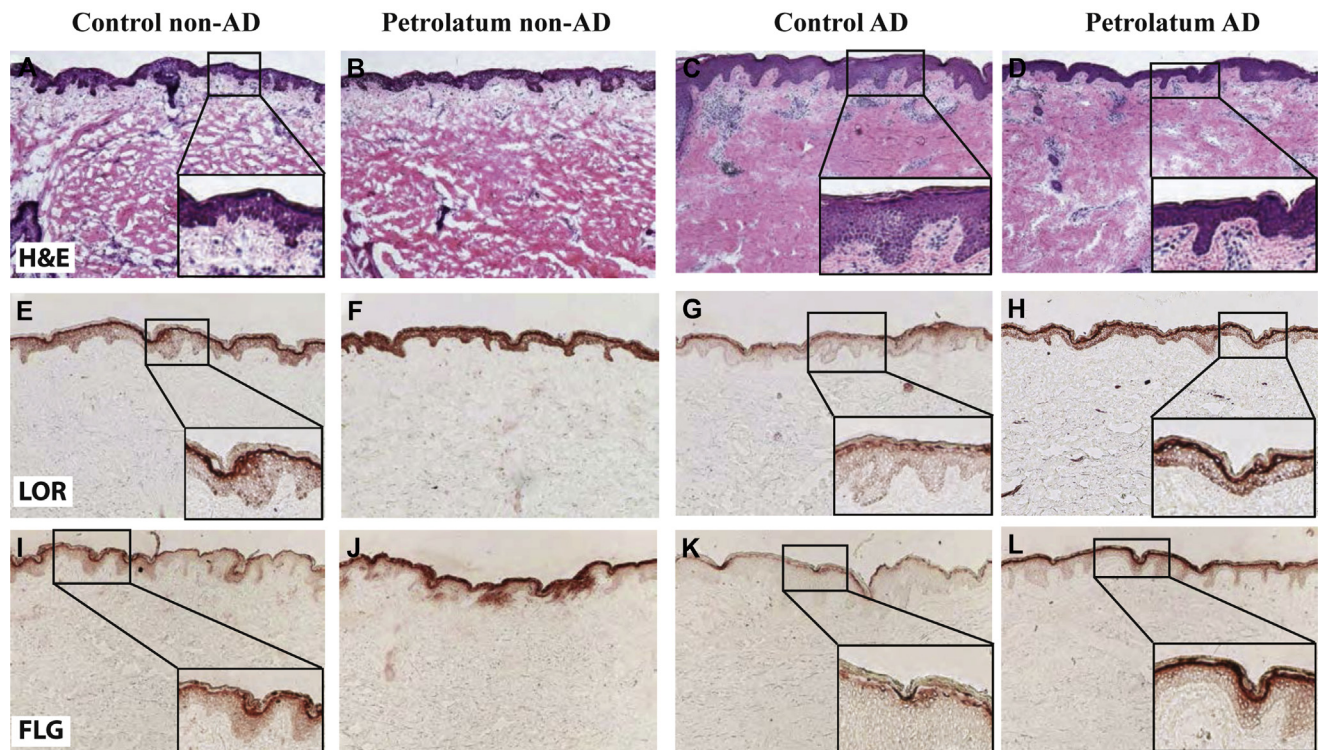


FIG 6. Representative staining of H&E (**A-D**), LOR (**E-H**), and FLG (**I-L**) in control skin and petrolatum-occluded skin from patients with AD and subjects without AD. AD skin shows parakeratosis and focal disruptions of the granular layer (Fig 6, **C**), with restoration of orthokeratosis with petrolatum (Fig 6, **D**). Weak and discontinuous LOR (Fig 6, **G**) and FLG (Fig 6, **K**) staining was observed in control AD skin, with increased intensity and restoration of continuous expression of both markers after occlusion with petrolatum (Fig 6, **H** and **L**; histologic magnification $\times 10$).

as determined by using IHC, and that psoriasin/S100A7, which acts against *E coli*⁶¹ and as an “alarmin” to amplify the skin’s inflammatory response to pathogens,⁶² also shows robust mRNA expression increases with petrolatum.

The IL-17 and IL-22 axes are responsible for fighting cutaneous infections in various disease states through induction of AMPs and innate immune genes.^{63–66} The AMPs found to be upregulated by petrolatum in our skin-profiling study were reported to be induced by IL-17 alone (HBD2/DEFB4A, LCN2, CCL20, and PI3)^{24,67,68} or both IL-17 and IL-22 (S100A7, S100A8, S100A9, and S100A12).⁶⁹ IL-17A, IL-22 and IL-23 (a known activator of the T_H17/T_H22 pathways)^{40,41,70} cytokines were also significantly upregulated with petrolatum occlusion compared with both occlusion alone and control skin or nonlesional AD skin, providing a potential explanation for increased AMP levels.

Innate immune genes (ie, *IL8*) were also significantly upregulated in petrolatum-occluded skin, which is consistent with their modulation by the T_H17 pathway.^{66,71} Importantly, petrolatum does not induce broad upregulation of all immune axes but rather is selective for T_H17 and T_H22 pathways, as suggested by the relative lack of induction of T_H2 cytokines (IL-13, IL-5, and IL-31) with petrolatum, particularly in the setting of nonlesional AD skin.

Because the nonlesional AD skin in our study already expressed increased levels of T_H2 cytokines and chemokines (IL-5, IL-10, and CCL26), as previously reported,¹⁶ we evaluated for differential gene modulation by petrolatum on patients with AD versus healthy subjects. Overall, lower magnitude responses were observed and fewer immune genes were differentially expressed in the petrolatum-occluded AD group compared with those in subjects without AD. Attenuation of genomic responses in patients with AD has also been observed in biopsy specimens from positive patch test skin reactions to common allergens, such as nickel and fragrance,⁷² suggesting an overall immune hyporesponsiveness in the setting of AD. It has been proposed that differential immune skewing and impaired T-cell priming in patients with AD can mitigate responses to certain cutaneous stimuli.^{33,73}

The changes observed with petrolatum are greatly dependent on baseline skin characteristics. An altered microbiome,^{74,75} reduced AMP levels,²⁴ cytokine axis deviation,³⁵ and a defective barrier^{68,76–78} are characteristic of AD and render these patients more susceptible to recurrent cutaneous infections, especially in comparison with psoriasis, a disease characterized by T_H17 skewing and increased AMP levels.^{21,79,80} Although our patients with AD mounted significant AMP responses with petrolatum occlusion, their responses were lesser in magnitude than those of the subjects without AD, possibly because of the upregulation of T_H2 markers, which have been shown to inhibit AMP production in nonlesional AD skin.^{22,24} In addition to its inhibitory effect on AMPs,¹² the T_H2 overexpression seen in nonlesional AD skin^{32,81} has also been suggested to reduce FLG mRNA expression and other structural components of corneocytes.^{82–86} Therefore the lack of T_H2 upregulation by petrolatum might be a major benefit when considering the potential use of petrolatum in patients with AD.

In addition to T_H2 skewing, a T_H1 deficiency is also present in AD skin,^{87,88} which has been associated with a lack of protective immunity against cutaneous viral infections, including eczema herpeticum.^{89,90} Our data show that petrolatum boosts the

T_H1/IFN- γ responses in patients with AD, potentially ameliorating a defective antiviral mechanism in patients who are prone to recurrent viral skin infections. Additionally, petrolatum occlusion resulted in reduced cellularity and strong induction of AMPs in AD skin, although to a lesser extent than in non-AD skin.

Patients with AD harbor structural barrier abnormalities, including reduced secretion of lamellar bodies in the SC,⁷⁶ altered function of the cornified cell envelope,³¹ and changes in composition of epidermal lipids.^{78,91,92} Petrolatum occlusion displayed marked changes in SC structure, with widening of the intercellular spaces leading to increased SC thickness. In accordance with our data, a prior report by Ghadially et al⁵ showed that petrolatum application induces cleft formation (intercellular petrolatum clumps) in the SC, leading to SC thickening, improved barrier function, and reduced TEWL. Future functional studies will need to determine the effect of petrolatum on barrier function, although recovery of consistent FLG and LOR expression throughout the epidermis of patients with AD suggests improvement in barrier integrity.

Emollient use has recently been shown to significantly reduce rates of AD development in high-risk newborns.^{93,94} Simpson et al²⁹ found that prophylactic emolliation reduced the relative risk of AD development by 50% in high-risk neonates, whereas a concurrent study also found a significant reduction in AD development with daily moisturizers in a Japanese population of high-risk neonates.³⁰ Although initial proposed mechanisms for the beneficial effect of emollients focused on barrier repair and decreased TEWL, recent preliminary data showed that early emolliation leads to an altered skin microbiome and skin pH in high-risk newborns.⁹⁵ Given our results showing increases in AMP levels, innate immune gene expression, and barrier characteristics, we offer a possible explanation for the preventive effect of emollients, which potentially alter the innate and adaptive immune profiles in axes relevant to protection against infections,^{96–100} AMPs, and barrier profile, ultimately changing the natural history of AD development in high-risk newborns.

The mechanism by which petrolatum mediates the above molecular and structural changes is yet to be determined. Recently, Van den Bogaard¹⁰¹ showed that coal tar, an ancient skin product used in the treatment of AD,¹⁰² mediates its effects through activation of the aryl hydrocarbon receptor (AHR) pathway. Although it is likely that most aryl hydrocarbons are separated from petrolatum during the process of its extraction from petroleum, trace amounts might still be present and might therefore mediate its effect through the AHR. Interestingly, AHR has been shown to transcriptionally regulate T_H17 and T_H22 cells, increasing IL-17 and IL-22 expression.^{103,104} This is in accordance with our results, which demonstrate significant induction of IL-17- and IL-22-regulated AMPs. Furthermore, a comparison of genes modulated by coal tar with genes modulated through petrolatum in our study showed that there are 2 overlapping genes, *TGM1* (transglutaminase 1) and *IVL* (involucrin), induced by both products (data not shown). Thus a possible mechanism for the effects of petrolatum might be AHR pathway activation, although this is speculative and requires further study.

Our study has several limitations. First, the presence of FLG or other skin barrier mutations might affect responses to petrolatum application. However, skin genotyping was beyond the scope of this study and should be addressed in the future.

Second, for practical reasons, we assessed petrolatum under occlusion only. Because petrolatum is more commonly used without occlusion, future studies will be required to determine molecular and barrier alterations induced by a nonoccluded petrolatum.

Lastly, future studies will need to assess and compare not only the long-term effect of emollients in high-risk newborns²⁹ but also the ability of different moisturizer categories to improve barrier function and AMP production. This is particularly important in preventing development of AD lesions and reverting the “abnormal” nonlesional AD phenotype^{16,105} back to a normal skin pattern.

Clinical implications: Because prophylactic moisturizers prevent AD development in high-risk newborns, our data can be further explored to evaluate the differential effects of various moisturizers on disease prevention, barrier function, and skin infections.

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METHODS

IHC

Biopsy specimens were frozen in optimal cutting temperature (O.C.T.) medium, and cryostat tissue sections of all patients were stained with hematoxylin (Fisher, Fair Lawn, NJ) and eosin (Shandon, Pittsburgh, Pa). IHC was performed with purified anti-human mAbs. For IHC, biotin-labeled horse anti-mouse secondary antibodies (Vector Laboratories, Burlingame, Calif) were used to detect the primary antibodies, and the staining signal was developed with chromogen 3-amino-9-ethylcarbazole (Sigma-Aldrich, St Louis, Mo). [Table E1](#) provides details on antibodies used for IHC. Epidermal thickness and positive cells per millimeter were manually quantified by using computer-assisted image analysis software (ImageJ 1.42; National Institutes of Health, Bethesda, Md).

Gene array analysis

RNA was extracted, and Affymetrix Human U133Plus 2.0 arrays (Affymetrix, Santa Clara, Calif) were used. Briefly, we used human HGU133Plus2.0 GeneChip probe arrays (Affymetrix) containing approximately 54,000 probe sets to assess expression of more than 47,000 transcripts, including approximately 38,500 genes and unigenes. Total RNA was extracted from tissues frozen in liquid nitrogen by using the Qiagen miRNeasy Mini Kit (Qiagen, Valencia, Calif). DNA was removed by using on-column DNase digestion with the Qiagen RNase-free DNase Set. Total RNA (100 ng) was reverse transcribed and amplified with Ovation Whole Blood Solution from NuGen (San Carlos, Calif). The labeled target was fragmented and hybridized to probe arrays with the Encore Biotin Module from NuGen. The chips were washed, stained with streptavidin-phycoerythrin, and scanned (HP GeneArray Scanner; Hewlett-Packard Company, Palo Alto, Calif). On each chip, the human housekeeping genes β -actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as controls. Suite 5.0 software normalized expression levels using these controls.

RT-PCR analysis

The TaqMan RT-PCR reaction was performed with EZ-PCR Core Reagents (Life Technologies, Grand Island, NY), according to the manufacturer's directions. The primers and probes for each gene are listed in [Table E2](#) and were designed by Life Technologies. *hARP*, a housekeeping gene, was used to normalize each sample and each gene. The primer sequences used were generated with the Primer Express algorithm (version 1.0; Applied Biosystems, Foster City, Calif) by using published genetic sequences (National Center for Biotechnology Information/PubMed), as follows: *hARP* forward, CGCTGCTGAACATGCTCAA; *hARP* reverse, TGTCGAA CACCTGCTGGATG; and *hARP* probe, 6-FAM- TCCCCCTTCTCC TTTGGGCTGG-TAMRA (Gene Bank accession no. NM-001002). Please see [Table E2](#) for details on primers and probes used. Data were analyzed and quantified by using software provided with the Applied Biosystems PRISM 7700 (Sequence Detection Systems, version 1.7).

Statistical methods

Statistical methods were applied to 2 different cohorts that were analyzed separately. The first cohort had IHC, microarrays, and RT-PCR

expressions taken from petrolatum-occluded and matched control skin from 29 patients. Data and their description are available on the Gene Expression Omnibus GSE60028 database repository. The second cohort contains RT-PCR gene expressions taken from control, occluded, and petrolatum-occluded skin measured in a set of 20 healthy volunteers. In this cohort gene expressions were normalized to *hARP* and multiplied by 10^5 . For the first 5 volunteers of this cohort, RT-PCR expressions were evaluated from 5 biopsy specimens: control, occlusion-only and occlusion with petrolatum at 48 hours, and occlusion-only and occlusion with petrolatum at 72 hours skin.

Microarray and RT-PCR analysis for the first cohort aimed to identify genes that were differentially expressed in petrolatum-occluded skin when compared with control skin. QC of microarray was carried out with standard QC metrics from Quality Control R package. Log₂-expressions were modeled by using linear mixed-effects models, with skin type as the fixed effect and a random effect for each patient. For microarray gene expression, the *limma* package in R version 3.1.0 was used to identify a set of 51 genes with FCHs of greater than 2 and a false discovery rate of less than 0.05 ([Table 1](#)). These DEGs are shown in the heat map in [Fig 2](#), followed by the estimated FCHs. The parameters for the hierarchic clustering in this heat map are as follows: McQuitty algorithm, Euclidean distance, and standardization by rows using the function *heatmap.2* in R package. Subgroup analysis for patients with AD and subjects without AD was carried out for a subset of 52 genes in which expressions were measured by using RT-PCR. Specification of the mixed-effects model considered as fixed effects: skin type (petrolatum-occluded or normal skin), AD personal history (yes/no), and their interaction. This model also included a random intercept for each subject. The package *nlme* was used for gene-wise estimation of the mean log₂-transformed expression and its SEM, which were associated with the following groups: control AD, control non-AD, petrolatum AD, and petrolatum non-AD. Restricted maximum likelihood estimated the log₂ FCHs for specific contrasts. A heat map with the same parameters as described before was created to illustrate the differences between the 4 groups. Blocks of interest in this heat map, followed by estimated FCHs and their statistical significance, are shown in [Fig 5](#). This mixed-effects modeling approach for subgroup analysis was also applied to IHC data, assuming normal distribution for cell counts. The contrasts of interest in IHC are the ones that compare cell counts in petrolatum-occluded with those in normal skin within the AD and non-AD groups.

Analysis for the second cohort consisted of fitting a linear mixed-effects model to log₂-transformed RT-PCR expressions of 17 genes (AMPs, innate immune, and cytokines). This model has only fixed effects for skin type (petrolatum-occluded, occluded, or normal skin) and a random effect for each subject. Pairwise comparisons after estimating the model parameters were evaluated with the use of the *estimable* function, which is part of the *gmodels* R package. The evaluation of differences between mean log₂-transformed RT-PCR expressions in the 5 different biopsy specimens taken from the first 5 volunteers was performed with ANOVA for repeated measures as implemented in Prism Version 6.0 software (GraphPad Software, La Jolla, Calif). *Post hoc* comparisons were carried out with paired *t* tests adjusting *P* values for multiple hypotheses. The results are displayed in [Fig E1](#).

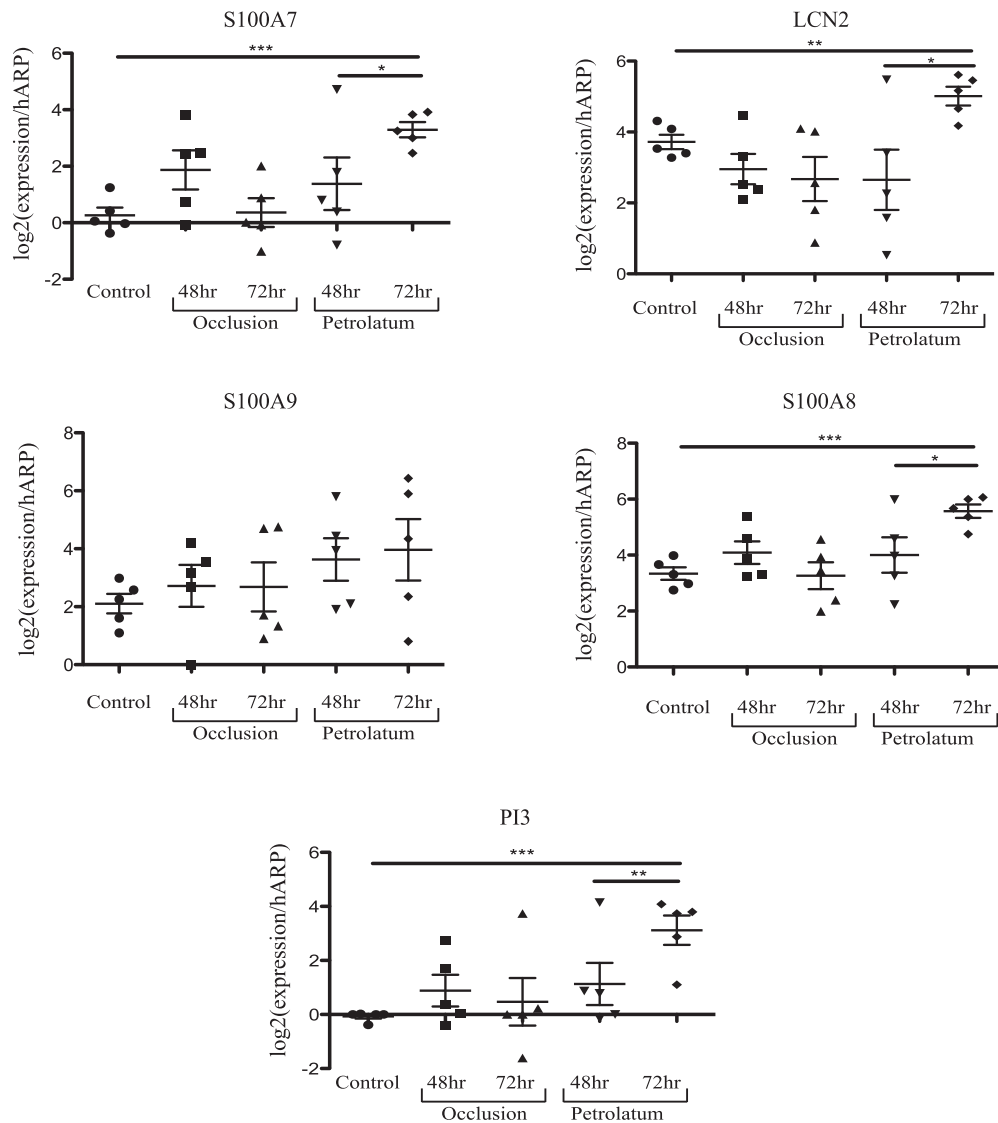


FIG E1. RT-PCR log₂ expressions normalized to *hARP* for 5 key AMPs. To assess the point of maximum gene induction for petrolatum and occlusion, we compared gene expression at different time points. *S100A7*, *LCN2*, *S100A8*, and *PI3* all showed significant increases in expression from baseline at 72 hours of occlusion with petrolatum but not at 48 hours. Bar plots represent means $\pm$ SDs. * $P < .1$, ** $P < .05$, and *** $P < .01$.

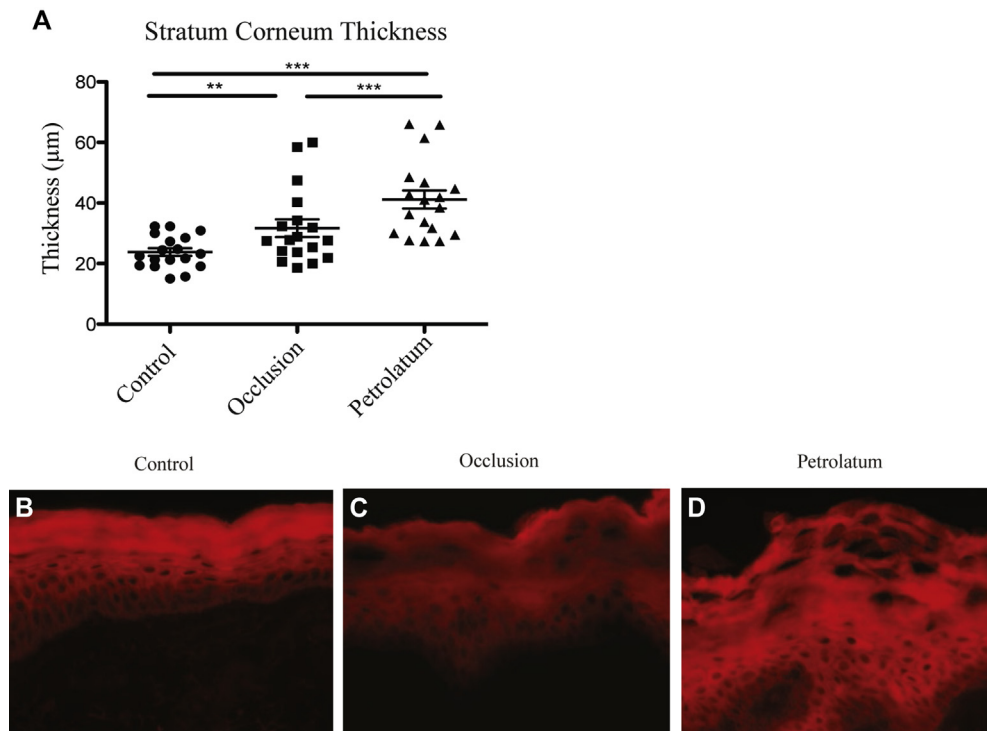


FIG E2. **A**, Thickness (in micrometers) of the SC in control, occlusion-only, and occlusion with petrolatum skin. Occluded and petrolatum-occluded SC showed significant thickness increases compared with baseline, and petrolatum also significantly increased SC thickness compared with occlusion alone. **B-D**, Immunofluorescence staining with Nile Red for neutral lipids in control (Fig E2, *B*), occlusion-only (Fig E2, *C*), and occlusion with petrolatum skin in a representative subject (Fig E2, *D*). Intercellular lipid clefts are observed after occlusion with petrolatum, which, in Fig E2, *B* and *C*, were not observed in control and occlusion-only skin, probably accounting for the increased SC thickness observed. Bar plots represent means $\pm$ SEMs. ** $P < .05$ and *** $P < .01$.

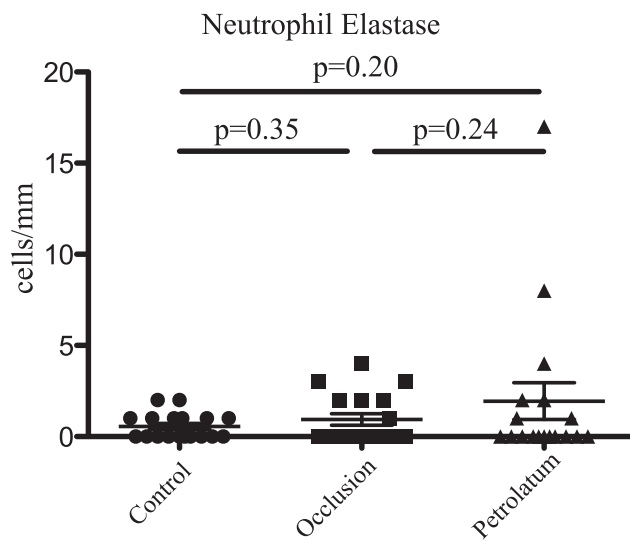


FIG E3. Neutrophil elastase cell counts of control, occluded, and petrolatum-occluded skin. There were no statistically significant increases in neutrophil elastase-positive infiltrates in occluded or petrolatum-occluded skin. Bar plots represent means $\pm$ SEMs.

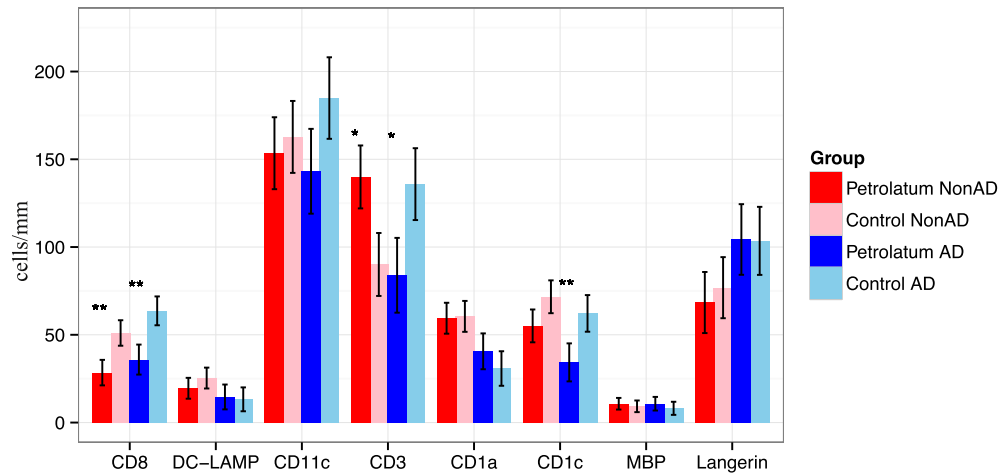


FIG E4. Cell counts for CD8, dendritic cell lysosome-associated membrane glycoprotein (*DC-LAMP*), CD11c, CD3, CD1a, CD1c, maltose-binding protein (*MBP*), and Langerin for control and occlusion with petrolatum skin in patients with AD and subjects without AD, demonstrating significantly decreased CD8 infiltrates for patients with AD and subjects without AD and decreases in CD1c and CD3 for patients with AD only. Bar plots represent means $\pm$ SEMs. * $P < .1$ and ** $P < .05$.

TABLE E1. Antibodies used for IHC

Antibody	Vendor	Clone	Dilution
S100A8/A9	Abcam	27E10	1:20
LCN2	Abcam	5G5	1:100
CCL20	LifeSpan BioSciences	B5917	1:20
FLG	Acris	FLG01	1:100
LOR	Abcam	polyclonal	1:200
Neutrophil elastase	Dako	NP57	1:200
Nile Red*	Sigma-Aldrich	N3013	500 µg/mL

*Used for immunofluorescence.

TABLE E2. Primers and probes for RT-PCR

Gene	Primer/probe
<i>IL1B</i>	Hs01555410_m1
<i>IL8</i>	Hs00174103_m1
<i>IL6</i>	Hs00985639_m1
<i>TNFA</i>	Hs01113624_g1
<i>IFNA1</i>	Hs00855471_g1
<i>HBD2/DEFB4</i>	Hs00823638_m1
<i>IL13</i>	Hs00174379_m1
<i>IFNG</i>	Hs00989291_m1
<i>IL17A</i>	Hs00174383_m1
<i>IL22</i>	Hs01574154_m1
<i>IL23A/IL23p19</i>	Hs00900828_g1
<i>IL12B/IL23p40</i>	Hs01011518_m1
<i>CCL27/CTACK</i>	Hs00171157_m1
<i>CXCL9</i>	Hs00171065_m1
<i>CCL20</i>	Hs01011368_m1
<i>PI3/elafin</i>	Hs00160066_m1
<i>LCN2</i>	Hs01008571_m1
<i>LL37</i>	Hs00189038_1
<i>S100A7</i>	Hs01923188_u1
<i>S100A8</i>	Hs00374264_g1
<i>S100A9</i>	Hs00610058_m1
<i>S100A12</i>	Hs00942835_g1
<i>TSLP</i>	Hs00263639_m1

TSLP, Thymic stromal lymphopoietin.